Biosynthesis of rhamnolipid by a Marinobacter species expands the paradigm of biosurfactant synthesis to a new genus of the marine microflora.

Microbial Cell Factories

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--Manuscript Draft--

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Abstract:	Background: In comparison to synthetically derived surfactants, biosurfactants produced from microbial culture are generally regarded by industry as being more sustainable and possess lower toxicity. One major class of biosurfactants are rhamnolipids primarily produced by Pseudomonas aeruginosa. Due to its pathogenicity rhamnolipid synthesis by this species is viewed as being commercially nonviable, as such there is a significant focus to identify alternative producers of rhamnolipids. Results: To achieve this, we phenotypically screened marine bacteria for biosurfactant production resulting in the identification of rhamnolipid biosynthesis in a species belonging to the Marinobacter genus. Preliminary screening showed the strain to reduce surface tension of cell-free supernatant to 31.0 mNm -1 . A full-factorial design was carried out to assess the effects of pH and sea salt concentration for optimising biosurfactant production. When cultured in optimised media Marinobacter sp. MCTG107b produced 740 ± 28.3 mgL -1 of biosurfactant after 96 h of growth. Characterisation of this biosurfactant using both HPLC-MS and tandem MS showed it to be a mixture of different rhamnolipids, with di-rhamnolipid Rha-Rha-C 10 -C 10 being the most predominant congener. The strain exhibited no pathogenicity when tested using the Galleria mellonella infection model. Conclusions: This study expands the paradigm of rhamnolipid biosynthesis to a new genus of bacterium from the marine environment. Rhamnolipids produced from Marinobacter have prospects for industrial application due to their potential to be synthesised from cheap, renewable feed stocks and significantly reduced pathogenicity compared to P. aeruginosa strains.
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Response to Reviewers:	Listed Response To Reviewer's Comments: MICF-D-19-00270 Reviewer #1: This study reported novelty about the identification of rhamnolipid biosynthesis in a species belonging to the Marinobacter genus after a phenotypic screening. The strain Marinobacter sp. MCTG107b exhibited no pathogenicity, proving to be promising for biosurfactant production. Useful information were obtained by the authors regarding the influence of salinity and pH in medium composition for biosurfactant production, extraction, and characterization of the produced biosurfactant. This paper contains enough new, significant and interesting information to deserve publication. However, the following issues must be addressed by a minor revision before its acceptance. 1. Changes in salinity per si can lead to changes in superficial tensions. Did the authors consider this? A simple test of superficial tension using the medium with the salinity concentrations used at the FFD could prove that this was not the source of the observed superficial tension reduction. In this way, the authors ensure that the superficial tension reduction is due only to biosurfactant production. Response: We thank the reviewer for positive comments about our manuscript. We have performed surface tension measurement of Zobell marine media with salinity concentrations used at FFD. We observed that salinity was not a source of the surface tension reduction. In our study, there was a marked fall in surface tension during the exponential growth phase coupled with a sustained low surface tension during stationary phase which indicated the biosurfactant produced by strain MCTG107b. This was further supported by gravimetric analysis of biosurfactant yield as well as quantitative analysis using HPLC-MS. 2. Should the results of reduction in superficial tension agree with the biosurfactant yield? Why the 3D response surface plots of both dependent responses are not coincident? Response: The results of reduction in superficial tension agree with the biosurfactant yield. The increase in salinity concentration influenced positively, in a statistically significant way, leading to higher biosurfactant yield (Fig 1A). However, the 3D response surface plots of both dependent responses are not coincident. Since the increase in salinity concentration influenced negatively, in a statistically significant way, the increase in biosurfactant production, leading to lower surface tension (Fig 1B). We have made appropriate changes in Results section. 3. In the biosurfactant production by Marinobacter sp. MCTG107b in bioreactor, when assessed gravimetrically, the mean biosurfactant yield obtained by liquid phase extraction and SPE purification of the cell-free supernatant volume was 740 mgL-1 (± 28.3 mgL-1). However, when Orcinol assays followed by HPLC-MS analysis were carried out on samples obtained from the bioreactor cultures the concentration of glycolipids obtained was 150 µgml-1 of culture. What can this difference be attributed to? What other compounds may be considered in gravitometry that were not detected as glycolipids by the Orcinol method? The authors could please include the explanation at the discussion topic. Response: Orcinol assay is a colorimetric method for the rapid indication of rhamnolipid in the fermentation broth. However, the limitation of orcinol method is that it is not specific for rhamnose and impurities present in the sample might interfere with the actual yield. Therefore, the method provides an inaccurate estimation of rhamnolipid concentration when compared to the purified rhamnolipid sample

measured gravimetrically.
We performed SPE purification of crude biosurfactant to remove unwanted fatty acids from the sample. SPE removed excess lipids from the sample which gave an absolute quantification of rhamnolipid yield. We followed the criteria suggested by Irorere et al. (2017) to quantify biosurfactant yield gravimetrically. The final yield of rhamnolipid was 740 ± 28.3 mgL-1 from the SPE purified sample. We have made appropriate changes in the Discussion section emphasising that.

Reviewer #2: The work by Tripati et al (MICF-D-19-00270) describes the isolation of a Marinobacter sp strain (MCTG107b) that produces rhamnolipids (RL) after the initial characterization of five Marinobacter isolates. The authors follow an optimization strategy to determine the pH (6.5) and salt concentration (30 g/L) conditions for RL production and characterized the congeners that this strain produces in this culture medium. This strain produces mainly di-RL (95%) and the most abundant congener is Rha-Rha-C10-C10 (52%).
I consider that this article is suitable for its publication in Microbial Cell Factories, but I suggest the following modifications before it is accepted for publication.

1. The authors report that Marinobacter MCTG107b produces 750 mg/L (measured gravimetrically) when cultured in a 5 L bioreactor using the optimized medium. It would be interesting that the authors provide for comparison, the yield of a P. aeruginosa strain under optimal conditions.

Response: We have added the information regarding the yield of P. aeruginosa in the Discussion section as suggested.

2. The authors test Marinobacter MCTG107b virulence in the Galleria mellonella model (which has been shown to posses an innate immune response and to yield similar results to the murine model), using Pseudomonas aeruginosa PAO1 strain as a positive control. In the Materials and Methods section the authors state that they use a bacterial suspension of a 1,000 CFU, which is within the range of the number of CFU that are used to test virulence, but in the description of the results presented in Fig. 4, (page 16 lines 396-397), they say that they use a similar inoculum as that used for PAO1 strain (around 20 CFU). If this is just a writing mistake it should be corrected, since non-pathogenic bacteria have been shown to be harmless at concentrations as high as 100,000 CFU per larvae (https://doi.org/10.3389/fmicb.2019.00311, https://doi.org/10.3389/fmicb.2019.01791). However, if Marinobacter MCTG107b was really injected at a low dose the experiment should be done again using at least 1,000 CFU.

Response: We thank the reviewer for pointing this out and as he/ she stated, this is a writing mistake. Based on a correlation of viable count and OD throughout the growth cycles, bacterial cultures (both 107b and PAO1) were diluted to a concentration of 5 X 104 CFU/ml, therefore providing a dose of 1000 CFU per 20 µL inoculum. This mistake has been corrected in both the Material and Methods and the Results sections of the revised manuscript. We also carried out experiments inoculating larvae with significantly higher doses of 107b, (up to 10,000 CFU), and observed the same level of survival. This point has been added to the Results section of the revised manuscript. However, as these experiments were carried out in the absence of a PAO1 group due to the fact that such a high dosage of P. aeruginosa would induce 100% mortality in the larva after a very short incubation period we did not include the data.

3. In the Discussion section the authors say that they were not able to detect rhlA, rhlB and rhlC genes using primers designed to amplify P. aeruginosa and Burkholderia genes, so they conclude that Marinobacter MCTG107b use enzymes with diverse sequences from known RhlA, RhlB and RhlC to synthetize RL. However the lack of PCR amplification of rhl gene homologs might be due to small differences in the sequence of the region of the primers used and not to the presence of genes coding for enzymes with "significantly different peptide sequences" in Marinobacter MCTG107b. Therefore, I consider that the lack of PCR amplification cannot be used as an argument to sustain the existence of non-homologous genes in Marinobacter MCTG107b.

Response: Since the drafting of this manuscript we have continued our investigations

into the presence of the RL biosynthesis genes within 107b. To do this we took the approach of designing multiple degenerate primers based on sequence alignments of published rhlA-C genes in both the Pseudomonas and Burkholderia genera. As yet we have been unable to amplify any fragment of DNA that has the potential to be an orthologue of these gene in 107b. Therefore, we stand by our conclusion that the biosynthesis of RL by this strain is being carried out by enzymes with significantly different sequences to that already known in both Pseudomonas and Burkholderia strains. Our work in this area is ongoing and is now aided by the possession of the complete 107b genome which, as we state in the manuscript, we are analysing bioinformatically. We hope to make the elucidation of the RL biosynthesis pathway within this strain the subject of a future publication. For now, we have amended the Discussion section of the revised manuscript to clarify our current findings and ongoing work around this issue.

4. In addition the authors argue that the production of Rha-C10 support their conclusion of a diverse RL biosynthesis pathway in Marinobacter MCTG107b, but RL containing only one fatty-acid moiety have been reported in P. aeruginosa cultures after long incubation periods and are much likely a product of degradation, which might be the case also with MCTG107b strain.

Response: We recognise that RL congeners possessing one fatty acid moiety have been reported in P. aeruginosa and that their presence may be due to degradation of congeners possessing the more common structure of two fatty acid moieties following a prolonged incubation period. However, our analysis of the congeners synthesised by 107b was not after a prolonged incubation period. Additionally, the percentage relative abundance of the Rha-Rha-C10 congener was at a level (5.13%) significantly higher than previously observed in P. aeruginosa. Finally, we also identified a product with a structure of Rha-Rha-C8 accounting for a relative abundance of 1.95%, however no congener with a structure of Rha-Rha-C8-C8 was observed. Taking these three points together we would rule out degradation as an explanation of the presence of these congeners with singular fatty acids and therefore stand by our initial conclusion.

5. In addition the authors sustain the involvement of horizontal gene transfer (HGT) of rhl genes into Marinobacter MCTG107b, based on the lack of detection of these genes by PCR, but I consider that the fact that this particular strain is unique in its ability to produce RL as compared with other Marinobacter isolates (Table 1) is sufficient evidence to support the involvement of HGT in Marinobacter MCTG107b ability to produce RL. The involvement of HGT in rhl genes inheritance by only some strains of a bacterial species has been discussed previously (Toribio et al., 2010).
The strategy of analyzing Marinobacter MCTG107b whole genome sequence to detect genes involved in RL biosynthesis is a straight forward approach, but I consider that the sequencing of other Marinobacter strains might not give information about the genes involved in the biosynthesis of this biosurfactant in Marinobacter, precisely because these genes seems to be inherited by HGT.
In summary, I consider that the Discussion section needs to be modified taking into account these points.

Response: We thank the reviewer for this very interesting assessment of the potential for HGT, however we do make this point using the reference Toribio et al., 2010 in the discussion section of the manuscript. In light of the reviewers comment we have modified our Discussion section with aim of making this point clearer. With regards to the WGS analysis pathway we are currently using, a point that is also mention in the Discussion section on the manuscript. Our plan is to compare the newly acquired genome of MCTG107b with the published genome sequences of other similar non-RL synthesising Marinobacter strains to detect regions of the genome that are either unique or significantly different and therefore might be due to HGT accounting for RL synthesis in this strain. We anticipate this work will form the basis of a future publication; therefore, we do not wish to elaborate further in this current manuscript.

Reviewer #3: Overall, the methodology in this study is well established and manuscript is carefully prepared.
There are still a few minor points to be addressed to improve the manuscript.

1. The writing of degrees Celsius (°C) should be corrected in the entire manuscript. It

	(°C) should be written separated from the previous number and correct sign should be used. Response: Revised as suggested. 2. The abbreviation for "liters" must be the same (l or L) throughout the manuscript. For example; in Line 169 it is written as ml; in Line 179 it is written as mL. Response: Revised, ml changed to mL. 3. In line 54 and 166: The references should be corrected Response: Corrected as suggested. 4. In Lines 193-201: No need to repeat the company name of rapeseed oil (Sigma-Aldrich) in all mention. Response: Revised as suggested 5. Line 261: Please correct the sentence as … At each time point, individual larva "was" recorded as either live or dead …. Response: Changed from were to was 6. Line 271: Please correct the sentence as …. accession numbers "shown" in Table 1…. Response: Made appropriate changes in Materials and Methods section. 7. Please mention why only 2 parameters (sea salt concentration and pH) for enhancement of biosurfactant production are selected when more parameters could be worked on simultaneously. Response: Several studies have reported that salinity and pH have major influence on the bacterial growth and biosurfactant production. The subtle changes in salinity and pH can exert an important effect on microbial communities in the marine environment. Therefore, we worked on these two important parameters for biosurfactant optimisation in marine isolate MCTG107b. Our FFD design showed biosurfactant production was increased at a pH 5.5-6.8 and sea salt concentration 22.5-45 gL-1 which indicated that the selected parameters have a significant effect on the biosurfactant production. We elected to further focus our study on the identification of the biosurfactant compound produced by novel marine isolate. We performed the characterisation of the rhamnolipid using HPLC-MS and Tandem-MS. Also, tested the non-virulence of the isolate in Galleria wax worm model.
Additional Information:	
Question	**Response**
Is this study a clinical trial?<hr>A clinical trial is defined by the World Health Organisation as 'any research study that prospectively assigns human participants or groups of humans to one or more health-related interventions to evaluate the effects on health outcomes'.	No

Biosynthesis of rhamnolipid by a *Marinobacter* species expands the paradigm of biosurfactant synthesis to a new genus of the marine microflora.

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Abstract

Background: In comparison to synthetically derived surfactants, biosurfactants produced from microbial culture are generally regarded by industry as being more sustainable and possess lower toxicity. One major class of biosurfactants are rhamnolipids primarily produced by *Pseudomonas aeruginosa*. Due to its pathogenicity rhamnolipid synthesis by this species is viewed as being commercially nonviable, as such there is a significant focus to identify alternative producers of rhamnolipids.

Results: To achieve this, we phenotypically screened marine bacteria for biosurfactant production resulting in the identification of rhamnolipid biosynthesis in a

species belonging to the *Marinobacter* genus. Preliminary screening showed the strain to reduce surface tension of cell-free supernatant to 31.0 mNm^{-1}. A full-factorial design was carried out to assess the effects of pH and sea salt concentration for optimising biosurfactant production. When cultured in optimised media *Marinobacter* sp. MCTG107b produced 740 ± 28.3 mgL^{-1} of biosurfactant after 96 h of growth. Characterisation of this biosurfactant using both HPLC-MS and tandem MS showed it to be a mixture of different rhamnolipids, with di-rhamnolipid, Rha-Rha-C_{10}-C_{10} being the most predominant congener. The strain exhibited no pathogenicity when tested using the *Galleria mellonella* infection model.

Conclusions: This study expands the paradigm of rhamnolipid biosynthesis to a new genus of bacterium from the marine environment. Rhamnolipids produced from *Marinobacter* have prospects for industrial application due to their potential to be synthesised from cheap, renewable feed stocks and significantly reduced pathogenicity compared to *P. aeruginosa* strains.

Key Words

Biosurfactant; Glycolipid; HPLC-MS; Marine bacteria; *Marinobacter*; Rhamnolipid

Background

Surfactant compounds possess both hydrophobic and hydrophilic moieties: they can modulate surface and interfacial tensions and are therefore widely utilised in a variety of different industries. Though many of these surfactant compounds are derived synthetically from petrochemical sources, numerous microorganisms have been shown to synthesise surfactant compounds. Surfactant compounds produced from a biological source are termed biosurfactants and are generally viewed as

being more sustainable and less toxic than their synthetically derived alternatives [1]. Marine microorganisms have been shown to be able to produce biosurfactants under extreme environments, caused by changes in salinity, increased UV exposure, limited nutrients, fluctuations in temperatures and pH [2-4]. Many marine bacterial species, commonly from oil-contaminated waters, have been reported to produce biosurfactants, and include members belonging to the genera *Alcanivorax*, *Alteromonas*, *Pseudoalteromonas* and *Halomonas* [5-7]. The biosurfactants they produce have the ability to solubilise hydrocarbons from the surrounding environment, which enhances the growth of indigenous bacteria capable of degrading aliphatic and polycyclic aromatic hydrocarbons (PAHs) [8]. These species therefore have tremendous industrial potential especially for application in microbial enhanced oil recovery (MEOR) and bioremediation purposes [7, 9, 10]. Furthermore, biosurfactant produced by psychrophilic marine bacteria are potentially exploitable in industrial processes for the preparation of biological detergents that are active at lower temperatures [11].

Particularly in the oligotrophic conditions of open ocean environments, marine bacteria have evolved to compete for the limited resources available to them. With respect to biosurfactants, they may be produced as secondary metabolites as, for example, to access hydrophobic growth substrates or to directly attack rival bacterial species competing for limited growth and energy sources [12]. In the latter case, biosurfactant compounds could be considered applicable for combatting pathogenic antibiotic-resistant microorganisms [13, 14]. Biosurfactants have also been shown to play an important role in biofilm development, the maintenance of biofilm structure and in substrate adhesion [15]. Abrogating the bacterium’s ability to produce a biosurfactant could therefore disrupt biofilm growth with a multitude of potential

applications including reducing infection risk to patients receiving implantable medical devices such as catheters [16, 17].

Biosurfactant compounds possess a wide array of molecular structures and are often classified based on their structure. One of the best studied groups of biosurfactants are the glycolipids, specifically rhamnolipids [10]. Rhamnolipids are composed of one or two rhamnose units linked in a 1, 2-glycosidic linkage to two *β*-hydroxy fatty acids (*β*-OH-FA or 3-OH-FA) ranging between 8-18 carbons in length. The most studied rhamnolipids are those produced by the Gram negative, opportunistic pathogen *Pseudomonas aeruginosa* [18]. Rhamnolipids have a broad range of potential applications in various industries, including for MEOR in the petroleum industry, as emulsifiers in the food and cosmetic industries, and as anti-microbial/ therapeutically-active agents in the pharmaceutical industry [19]. Despite their versatile potential industrial applications, the exploitation of rhamnolipids has been limited due to the pathogenic nature of *P. aeruginosa*. To overcome this there has been an increased interest in the discovery of non-pathogenic rhamnolipid producers. Recent reports have shown rhamnolipid production by non-pathogenic species of *Pseudomonas*. For example, a non-pathogenic rhamnolipid-producing marine *Pseudomonas* sp. MCTG214(3b1) was shown to produce both mono and di-rhamnolipids [20]. An arctic marine bacterium identified as *Pseudomonas fluorescence* species was reported to synthesise five mono-rhamnolipid congeners [21]. Outside of the *Pseudomonas* genus, rhamnolipids have been shown to be synthesised by a number of non-pathogenic species of the genus *Burkholderia* [22, 23]. Discovery and isolation of novel non-pathogenic rhamnolipid producers is an attractive route to compete with the synthetic surfactants and meet future global biosurfactant requirements. Global estimates of microbial cell abundances in

seawater range from 10^4 to 10^7 cells per millilitre, with an estimated average taxonomic diversity of 1000 species per millilitre [24]. Collectively, this offers a significant opportunity to discover novel biosurfactant producers, including that produce rhamnolipids, and that are non-pathogenic [25]. Importantly for commercial exploitation, it is essential that the economics underlying the production of the biosurfactants is viable and able to compete with chemically-derived surfactants in the global market. This needs to be achieved by reducing manufacturing costs and enhancing fermentation yields. There are numerous factors for optimising the process of biosurfactant production, including optimising the composition of the culture medium, pH, dissolved oxygen levels during growth, culture agitation and incubation temperature. Statistical design of experiments (DoE) methods, such as full factorial design (FFD), have been shown to be an efficient and useful method to optimise biosurfactant production using a reduced number of experiments [26-28].

In this study, we investigated biosurfactant production in five bacterial strains isolated from coastal and offshore sites in the USA, Scotland and Norway, and all phylogenetically identified to belong to the genus *Marinobacter*. One of these strains, *Marinobacter* sp. MCTG107b, possessed phenotypic traits indicative of biosurfactant synthesis. The bioprocess factors for biosurfactant production in this strain were optimised for maximum production yield in shake-flask culture using FFD. Using high performance liquid chromatography-mass spectrometry (HPLC-MS) and tandem-MS, the chemical structure of the biosurfactants produced by this strain were analysed and confirmed to be rhamnolipids. This study reports the first description of rhamnolipid production by a *Marinobacter* and, importantly also, extends the paradigm of rhamnolipid production to a new bacterial genus which is recognised as ubiquitous in the marine environment and commonly associated with oil spills.

Materials and Methods

Strains and culture conditions

The marine bacteria used in this study were isolated from surface seawater samples collected from offshore location in the USA, UK and Norway (Table 1). The method of isolation has been previously described by Twigg *et al* [20]. Following isolation, these strains were routinely cultured at 30 °C ~~°C~~ in ZM/1 medium which consists of 30 gL^{-1} sea salts (*Sigma-Aldrich*), 5 gL^{-1} Bacto Peptone (*BD Biosciences*), 1 gL^{-1} yeast extract (*Sigma-Aldrich*) and supplemented with trace elements and vitamins after autoclaving [29]. *P. aeruginosa* strain PAO1 was purchased from the *ATCC* (ATCC 15692) and was cultured at 37 °C ~~°C~~ in Nutrient Broth (*Oxoid*). Solid media plates used in this study were composed of appropriate culture media supplemented with 1.5% (w/v) agar (*Sigma-Aldrich*).

Phylogenetic identification

Genomic DNA (gDNA) was extracted from approx. 1×10^8 bacterial cells via a DNeasy Blood and Tissue Kit (*Qiagen*) used as per the manufactures instructions for Gram-negative bacteria. Extracted gDNA was quantified and assessed for purity by measuring absorbance at 260 nm and 280 nm using a Nanodrop 2000 spectrophotometer (*Thermo Fischer*). The 16S rRNA gene was then amplified using the Polymerase Chain Reaction (PCR) with the universal primers 9bfm and 1512uR. PCR reactions contained 50 ng of gDNA, 1.5 mM $MgCl_2$, 1 × PCR buffer (*Thermo Fischer*), 0.2 mM dNTP mix (*Thermo Fischer*), 0.5 mM of each primer (*Thermo Fischer*), and 2 U of *Taq* DNA polymerase (*Thermo Fischer*). The PCR reaction was as follows: one cycle initial denaturation at 94 °C for 3 min; 30 cycles of denaturation

at 94 °C for 45 sec; annealing step at 52 °C for 30 sec; extension step at 72 °C for 90 sec; and one cycle final extension at 72 °C for 5 min. Following amplification, PCR products were separated on a 1% (w/v) agarose gel made with TBE buffer (*Thermo Fischer*), and amplicons of approx. 1.5 kb were subsequently purified from the gel using a Wizard SV Gel and PCR Clean Up System (*Promega*). Amplified 16S rDNA was quantified and assessed for purity as above. The purified 16S rDNA was sequenced using the Sanger method by *Eurofins Genomics* (Cologne, Germany) with primers 9bfm, 536F, 907R and 1512uR [30, 31]. The resultant DNA sequences were compared to the NCBI nucleotide database using BLASTn.

Phenotypic screening for biosurfactant production

The five marine bacterial strains were screened for their ability to reduce surface tension and to emulsify oil in water. Bacterial cultures were centrifuged at 13,000×*g* for 15 min and the supernatant fractions (in triplicate) used to perform surface tension measurements at room temperature (21 °C~~°C~~) according to the Du Noüy ring method using a K10ST A KRÜSS KIOT Tensiometer (*Krüss*) [32]. The surface tension of sterile ZM/1 media supplemented with 1% (v/v) rapeseed oil (*Sigma-Aldrich*) was also measured as a comparative control. To evaluate for emulsification, the emulsification index (EI) of the supernatant fractions was measured by adding 2 mL~~l~~ of the supernatant to an equal volume of kerosene and vortexing at high speed for 2 min. The stability of resultant emulsions was observed after 24 h settlement. The EI_{24} (i.e. EI after 24 hours) was calculated as a percentage of the height of the emulsified layer to the total height of the liquid prior to emulsification by vortexing [33]. As a control for comparison, sterile ZM/1 medium was used.

Optimization of growth conditions

The growth of MCTG107b in different physical and media conditions was investigated. For this, shake flask experiments were carried out in 1 L Erlenmeyer flasks containing 90 mL of ZM/1 medium and inoculated (10% v/v) with a seed culture grown under the standard conditions described earlier. For the carbon source, glucose 1% (w/v) final concentration was used in all experiments. All flasks were incubated in a rotary orbital incubator set at 200 rpm. However, physical and media conditions tested included various concentrations of sea salts (5, 10, 20, 30 and 40 gL^{-1}), temperature (25, 28, 30 and 37 °C~~°C~~) and pH (4.0, 5.5, 7, 8.5). Samples of the culture medium in these various experiments were taken at various time points for optical density (OD) measurements at 600 nm to monitor the growth of the cells.

Optimisation of growth media for biosurfactant production

The effect of sea salt concentration and pH for enhancement of biosurfactant production by MCTG107b was carried out by FFD, using surface tension, (measured as described previously), and biosurfactant yield, (measured gravimetrically), as response variables. These experiments were carried in shake flask culture using ZM/1 medium supplemented with 1% (v/v) rapeseed oil ~~(*Sigma-Aldrich*)~~ with various concentrations of sea salts (5 to 40 gL^{-1}) and pH (5.5 to 8.5) according to the experimental designs (Supplementary Table 1). In total, 14 experiments were performed (2^2 FFD with 8 assays and 6 replicates at the centre point).

Biosurfactant extraction and purification

Biosurfactant compounds were extracted and purified from 3-L cultures of strain MCTG107b when grown in optimised ZM/1 medium supplemented with 1% (v/v) rapeseed oil ~~(*Sigma-Aldrich*)~~ using a 5.0 L Biostat B bioreactor (*Sartorius Stedim*) equipped with a mechanical foam separator. The reactor vessel was inoculated (10% v/v) with a MCTG107b seed culture grown to exponential phase in ZM/1 supplemented with 1% (w/v) glucose at 30 °C and 200 rpm. Internal temperature of the culture was maintained at 30 °C~~°C~~ throughout the growth cycle. Stirrer speed and aeration varied between 300 and 600 rpm in order to maintain DO_2 levels at 50%. Cultures were incubated for 96 h; during growth, dissolved oxygen and pH were continually monitored and samples were taken at 24 h intervals to monitor growth and BS.

At the termination of the culture (96 h), the biosurfactants were extracted using liquid phase extraction. For this, the culture volume was first centrifuged (13,000×*g*; 15 min) and then the supernatant collected and acidified to pH 2.0 with 1M HCl (*Sigma-Aldrich*) prior to extraction three times with an equal volume of ethyl acetate (*Sigma-Aldrich*). The organic phase was separated and dried using $MgSO_4$ (*Sigma-Aldrich*), filtered and rotary evaporated under vacuum at 40 °C to obtain a crude extract [34]. Crude extracts were then purified by Solid Phase Extraction (SPE) using Strata SI-1 Silica (55 µm, 70 Å) Giga tubes (*Phenomenex*). Purified BS extracts were gravimetrically assessed and stored at 4 °C for further analysis [35].

Chemical analysis of biosurfactant compounds

Biosurfactant compounds extracted from strain MCTG107b were initially analysed using the orcinol method [34]. To each 100 µL sample, 900 µL of a solution

containing 0.19 % (w/v) orcinol (*Sigma-Aldrich*) in 53 % H_2SO_4 (*Sigma-Aldrich*) was added. Samples were then heated to 80 °C for 30 min, after which the samples were cooled to room temperature. The absorbance of the samples was measured at 421 nm. The concentration of glycolipid present in the samples were calculated to those generated using a standard rhamnose at concentrations of 0-100 $\mu g mL^{-1}$ [36].

Individual biosurfactant congeners were identified in the SPE purified extract by a UHPLC system with RS Diode Array detector (*ThermoFisher Scientific*) in conjunction with the amaZon SL dual funnel Ion Trap spectrometer LCMS system (Bruker). An analytical column of Acclaim RSLC, 120 C18, 2.2 µm 120Å (2.1 x 100 mm) (*ThermoFisher Scientific*) was used for analysis. The gradient elution sequence used was as follows: 20% B to 100% in 30 min, 100% B for 10 min, 20% B in 5 min. Solvents A= H_2O (0.1% TFA), B=AcCN (0.1%TFA). The sample injection volume used was 10 µL. Spectra were acquired in the positive mode from *m/z* 200 to 2000. Tandem-MS was performed using Thermos System LC P4000 (*ThermoFisher Scientific*) coupled to a LCQ classic MATT ion trap mass spectrometer (*ThermoFisher Scientific*) equipped with a 150 X 4.6 mm Kinetex 5 µM F5 100 Å LC column. HPLC-grade water and analytical-grade acetonitrile were used as mobile phase. The sample injection volume was 5 ~~µl~~ µL and the spectra were acquired in the negative mode from *m/z* 175 to 700. The fragmentation of the molecules was done with helium gas at the normalised 40% collision energy with the activation q value of 0.25.

Galleria mellonella infection model

Virulence assessment of strain MCTG107b was carried out using the *G. mellonella* infection model (Hill *et al.* 2014). A comparative positive control for these experiments was *P. aeruginosa* PAO1 (ATCC 15692). Using the Miles and Misra method [37], viable count (CFU) and OD 600 nm were correlated throughout the growth cycles of both strains. Following this, 10 mL ~~Ten millilitres~~ of stationary phase culture was centrifuged (10,000×*g*; 20 min) and the pelleted cells washed in sterile phosphate buffered saline (PBS). The washed cells were re-suspended to OD 600 nm 0.4 in PBS to a concentration of 5×10^4 $CFUmL^{-1}$. ~~Using the Miles and Misra method [37], the CFU count at OD 600 nm of 0.4 was established and the samples diluted in sterile PBS to a concentration of 1×10^3 $CFUml^{-1}$.~~ *G. mellonella* larvae (*Pets at Home*, Belfast) of approx. 20 mm in length and 200 mg in weight were selected and 20 ~~µl~~ µL of either bacterial sample (1000 CFU) or PBS (negative control) was injected into the posterior pro-leg of individual larvae (n=10 per experimental group). Injection was carried out using a 0.30 mm (30G) x 8 mm hypodermic needle (BD). Immediately following injection, the larvae were incubated at 37 °C and observed at set time points during the course of a 48 h period. At each time point, individual larva ~~were~~ was recorded as either live or dead. The experiment was performed on three independent occasions which gives a total of n=30 per experimental group [20, 38].

Statistical analysis and data availability

Statistical analysis of bacterial growth experiments was carried out in GraphPad Prism V.7 using a one-way ANOVA followed by Tukey's post-hoc testing; the significance of the results was tested at $p < 0.05$ level. The data obtained from FFD experiments were subjected to statistical analysis by TIBCO Statistica software version; the significance of the results was tested at $p < 0.05$ level. All sequence data

was submitted to GenBank (NCBI, USA) and the assigned accession numbers of strains are given ~~shown~~ in Table 1.

Results

Strain identification and initial phenotypic screening

Five marine bacterial isolates – MCTG107b, MCTG4b, MCTG106, MCTG167 and MCTG161(2c3) – were investigated for biosurfactant production. BLASTn analysis of partial 16S rRNA gene sequences from these strains showed >99% similarity to the genus *Marinobacter* (Table 1). Following phenotypic screening, strains which reduced the surface tension of culture medium to below 35 mNm^{-1} and/or produced a stable emulsion after 24 h were considered as potential biosurfactant(s) producers (Table 1). Strain MCTG167 was unable to form a stable emulsion or to significantly reduce the surface tension when compared to un-inoculated medium controls (58 mNm^{-1}). Strain MCTG106 and MCTG161(2c3) also showed no significant reduction in surface tension, however both strains were able to form stable emulsions after 24 h. Strain MCTG4b emulsified kerosene with an EI_{24} of 40%, whereas it reduced the ST of supernatant fractions to 38.5 mNm^{-1}. Strain MCTG107b showed the highest surface activity, significantly reducing the surface tension of the culture broth to 31 mNm^{-1}, and producing stable emulsions with kerosene (EI_{24} of 40%). Therefore, *Marinobacter* sp. MCTG107b was selected for further study.

Bacterial growth of *Marinobacter* sp. MCTG107b

To determine optimal conditions for the growth of *Marinobacter* sp. MCTG107b, and with a view to optimising its production of biosurfactant, growth was monitored in different physical and media conditions that included evaluating different salinities,

pH, temperatures and nitrogen sources. *Marinobacter* sp. MCTG107b grew optimally at a range of salinity concentrations, from 5.0 gL^{-1} to 40 gL^{-1} sea salts. In medium containing no added sea salts, no growth was observed. Strain MCTG107b grew optimally within a pH range of 5.5 to 8.5, whereas it was significantly inhibited under more acidic conditions (pH 4.0). The strain grew optimally at temperatures ranging from 25 °C to 37 °C (Fig S1).

Optimization of culture conditions for biosurfactant production

To optimise the media composition for maximal biosurfactant production by strain MCTG107b, the effects of salinity and pH, as well as the interaction between these variables was assessed by applying FFD, 2^2. Compared to pH, salinity was observed to be the most important factor affecting the reduction of surface tension when this was measured for cell-free culture supernatant fractions. The surface tension was found to vary between 30.5 mNm^{-1} to 40.2 mNm^{-1} (Table S1). Experimental results were used to generate two equations that modelled the relation between pH/ salinity and the outputs biosurfactant yield and surface tension. According to the response values obtained from the designed experiments, the following regression equations were obtained for both biosurfactant yield [1] and surface tension [2]:

$$Yield = 22.17262 + 39.88095 * pH(1) + 36.60714 * Salts(2) - 4.64286 * pH * Salts(1\ by\ 2) \quad [1]$$

$$Surface\ Tension = 50{,}03036 - 1{,}70714 * pH(1) - 0{,}55690 * Salts(2) + 0{,}05476 * pH * Salts(1\ by\ 2) \quad [2]$$

Significance of the present model was validated through analysis of variance ($p \leq 0.05$) (Table S2). The observed values for biosurfactant yield were significantly close to

those determined by the model (R^2 = 0.996). The observed and the predicted values for surface tension modelled here also demonstrated that the experimentally observed values were significant to those determined by the model (R^2 = 0.958). Results from the FFD analysis for the outputs of biosurfactant yield and surface tension were expressed as 3D response surface plots showing the relationship between independent and dependent variables (Fig 1). Increased salinity significantly and positively influenced biosurfactant yield. Our model demonstrated a significant relationship between biosurfactant yield and the two variables tested (salinity and pH). We modelled an increased biosurfactant yield when pH was low and salinity was increased from the central to the highest level. ~~(Fig 1A).~~ The increase in salinity positively influenced biosurfactant yield, in a statistically significant way (Fig 1A). Similarly, t~~T~~he model ~~also~~ showed that an increase ~~changes~~ in pH did not cause major impacts on surface tension, whereas an increase in salinity from the central to highest point led to a decrease in surface tension. The results of reduction in superficial tension agree with the biosurfactant yield. However, the 3D response surface plots of both dependent responses are not a coincident. ~~(Fig 1B).~~ Since, the increase in salinity concentration negatively influenced, in a statistically significant way, the increase in biosurfactant production, leading to lower surface tension (Fig 1B). These data demonstrate salinity and pH are critical factors that markedly affected the production yield of biosurfactant. Our model showed maximum production of biosurfactant at a pH 5.0–6.8 and salinity 22.5–40.0 gL^{-1}, resulting in a predicted yield of between 460–800 mgL^{-1}. The optimised medium with a sea salt concentration of 30 gL^{-1} and pH 6.5 was therefore chosen for all subsequent experiments with strain MCTG107b.

Growth and biosurfactant production by *Marinobacter* sp. MCTG107b in a bioreactor

Based on our results above, an optimised culture medium was used to produce biosurfactant from *Marinobacter* sp. MCTG107b employing a bench-scale 5.0 L bioreactor. Biomass, culture pH and surface tension were monitored continually throughout the growth cycle. The cell concentration at the time of inoculation (t = 0 h) was 2.02×10^{6} CFUml^{-1}. An exponential growth phase was maintained for the first 24 h, followed by a stationary phase from 24 h to 96 h, and the cell concentration reaching 6.46×10^{9} CFUml^{-1} by the end of the fermentation (Fig 2). The pH of the culture was observed to fall during the course of the exponential growth phase and then remained moderately constant during the stationary phase. A similar pattern was observed for the surface tension that was measured for cell-free supernatant samples over the course of the growth phase. The strain achieved the lowest surface tension value (31 ± 0.7 mNm^{-1}) after 24 h of fermentation and then remained almost constant until the end of the fermentation (Fig 2). When assessed gravimetrically, the mean biosurfactant yield obtained by liquid phase extraction and SPE purification of the cell-free supernatant volume from replicate 96 h bioreactor cultures of *Marinobacter* sp. MCTG107b was 740 mgL^{-1} (± 28.3 mgL^{-1}).

Chemical characterisation of the biosurfactant produced by *Marinobacter* sp. MCTG107b

The traits measured for biosurfactant production (i.e. surface tension reduction and emulsification) were indicative that *Marinobacter* sp. MCTG107b produces a glycolipid biosurfactant. To confirm this, Orcinol assays followed by HPLC-MS analysis were carried out on samples obtained from the bioreactor cultures. Orcinol

assays performed with cell-free supernatant samples indicated the presence of glycolipids at 150 µgmL^{-1} of culture. The production and presence of glycolipids was further investigated by mass spectrometric analysis. The identification of glycolipid congeners produced by strain MCTG107b was characterized by HPLC-MS operating in the positive mode. The observed products possessed *m/z* values that corresponded to values for known rhamnolipids, indicating that the biosurfactant synthesised by strain MCTG107b was a mixture of rhamnolipid congeners. We identified a variety of separate rhamnolipid congeners present in purified cell-free supernatant extracts from culture samples of the strain (Table 2). These congeners included both mono- and di-rhamnolipids; however, there was an overwhelming preference toward the synthesis of di-rhamnolipid (95.39% of total rhamnolipid abundance). The congener with the highest relative abundance (52.45%) possessed an *m/z* value of 651.73. This value correlated with *α-L*-rhamnopyranosyl-*α-L*-rhamnopyranosyl-*β*-hydroxydecanoyl-*β*-hydroxydecanoate (Rha-Rha-C_{10}-C_{10}) with a molecular weight of 650.79 Da. The next most abundantly synthesised congeners were Rha-Rha-C_{10}-$C_{10}CH_3$ (23.07%), Rha-Rha-C_{10} (5.13%), Rha-Rha-C_{10}-C_{12} (5.01%), Rha-Rha-C_{10}-$C_{12}CH_3$ (3.26%) and Rha-$C_{14:2}$ (3.18%) (Table 2). As rhamnolipid production has not been previously observed in any member of the *Marinobacter* genus, these data were further investigated for confirmatory evidence of this. For this, tandem-MS was performed on the major molecular ion shown to be synthesised by strain MCTG107b. Tandem-MS analysis of the compound with an *m/z* of 651.73 revealed the detection of ‘daughter’ ions with molecular weights indicative of the fragmentation of Rha-Rha-C_{10}-C_{10} (Fig 3). These data, together with the phenotypic results and initial HPLC-MS and Nuclear Magnetic Resonance (NMR)

spectroscopy analysis (data not shown), confirms the synthesis of rhamnolipid by *Marinobacter* sp. MCTG107b.

Assessment of virulence using the *Galleria mellonella* infection model

The potential virulence of *Marinobacter* sp. MCTG107b was assessed and compared to that of the rhamnolipid producing opportunistic pathogen *P. aeruginosa* using the *G. mellonella* infection model. *P. aeruginosa* PAO1 killed 100% of the infected larvae 24 h post inoculation with as little as ~~20~~ 1000 CFU. When larvae were inoculated with an equal CFU count of *Marinobacter* sp. MCTG107b or with sterile PBS, the larvae showed 97% survival 48 h post inoculation, with only one larva dying in each experimental group at 22 and 20 hours post inoculation respectively (Fig 4). Similar survival rates were also observed when larvae were inoculated with significantly higher doses of *Marinobacter* sp. MCTG107b (up to 10,000 CFU in 20 µL~~µl~~) (data not shown).

Discussion

Marine bacteria are reported to secrete surface-active molecules that can interact with hydrocarbons to increase the emulsification of the hydrocarbon molecules in seawater to enable these, and also non-biosurfactant producing bacteria to access these molecules for uptake and use as a source of carbon and energy [39, 40]. *Marinobacter*, a genus of *Gammaproteobacteria*, has previously been shown capable of utilising hydrocarbons as growth substrates by producing biosurfactants or bioemulsifier [41, 42]. In the present study, five marine bacterial strains, which were originally isolated for their ability to grow on and degrade PAHs, were identified to belong to the genus *Marinobacter* based on 16S rDNA gene sequencing. These isolates were screened to evaluate their potential as biosurfactant producers when

cultivated in marine media using rapeseed oil as a carbon source. Many previous studies have reported that the inclusion of peptone in marine media is essential for biosurfactant synthesis [43, 44]. Here we show that in response to adding rapeseed oil to peptone containing ZM/1 media, these five *Marinobacter* strains displayed varying phenotypic responses which were indicative of biosurfactant synthesis. Phenotypic comparison of all these strains showed that *Marinobacter* sp. MCTG107b, isolated from sea surface water samples off the coast of Oregon, USA, showed maximum reduction in the surface tension of cell-free supernatant fractions. As the ability to reduce the surface tension of cell-free supernatant is a key phenotypic marker of low-molecular weight biosurfactant synthesis, this strain was selected for further investigation [45].

Considering that subtle changes in salinity and pH can exert an important effect on microbial communities in the marine environment [46, 47], these parameters were tested for their influence on the growth of strain MCTG107b and its production of biosurfactant. Indeed, several studies have shown the effect of these two parameters on bacterial biosurfactant synthesis. For example, *Bacillus subtilis* N3-1P, isolated from brewery waste, reduced the surface tension of the culture medium to the greatest extent at a pH of 6.41 when compared to a range of other media pHs [48]. A thermophilic and halo-tolerant strain of *P. aeruginosa*, isolated from oil-contaminated soil, produced biosurfactant when cultured in media containing a salinity range of 0–6% (w/v) [49]. In contrast *B. licheniformis* BAS50, isolated from a deep oil well, produced biosurfactant when using a salinity of up to 13% NaCl, which is a salinity equivalent to that present in many petroleum reservoirs [50]. Based on these previous studies, we carried out FFD modelling to identify optimal concentrations of salinity and pH for the maximal production of biosurfactant by

strain MCTG107b, and which revealed that a pH range of 5.0–6.8 and salinity concentration of 22.5–40 gL^{-1} were optimal. Interestingly, while salinity was the dominant factor affecting the reduction of surface tension, the strain preferred an acidic pH for increased biosurfactant production. This was at the expense of bacterial growth, indicating that when the culture medium was alkaline, cells were directed to the production of cellular biomass over biosurfactant synthesis. Whilst not within the focus of this study, future work could be directed to explore this pH-mediated biosurfactant response and whether it is transposable to the global ocean in order to predict microbial biosurfactant production under future climate change conditions, such as ocean acidification. The profile of a marked fall in surface tension during the exponential growth phase coupled with a sustained low surface tension during stationary phase indicated the biosurfactant produced by strain MCTG107b is likely a secondary metabolite, as has been similarly reported for *P. aeruginosa* [51], *B. thailendensis* [23] and marine *Pseudomonas sp.* MCTG214(3b1) [20].

Orcinol assay yielded 150 μgmL^{-1} of rhamnolipid in the cell free supernatant. Orcinol assay is a colorimetric method for the rapid indication of rhamnolipid in the fermentation broth. However, the limitation of orcinol method is that, it is not -specific for rhamnose and impurities present in the sample might interfere with the actual yield. We followed the criteria suggested by Irorere *et al.* (2017) to quantify biosurfactant yield gravimetrically. The final yield of rhamnolipid was 740 ± 28.3 mgL^{-1} from the SPE purified sample. SPE purification of crude biosurfactant removed excess lipids from the sample which gave an absolute quantification of rhamnolipid yield. Similarly, Perfumo *et al.* (2013) reported purified rhamnolipid yields in the range of 0.8−1.7 gL^{-1} from *P. aeruginosa* strains. While, the orcinol assay provided an overestimation of rhamnolipids at 7.7−9.5 gL^{-1}.

The chemical characterisation of the purified biosurfactant synthesised by strain MCTG107b was achieved using HPLC-MS – a methodology demonstrated to be the most effective for identifying biosurfactant compounds [35], as described in a recent review by Irorere *et al.* (2017). We identified 14 separate rhamnolipid congeners with Rha-Rha-C_{10}-C_{10} (*m/z* 651) being the most abundant (52.45%). Furthermore, elucidation of this major molecular ion synthesised by *Marinobacter* sp. MCTG107b was performed by tandem-MS. When fragmented by MS-MS, the parent ion showed characteristic fragments at *m/z* 479 and *m/z* 339, which were in agreement with the previous tandem-MS analysis of Rha-Rha-C_{10}-C_{10} performed by Zhao *et al*. [52]. The pattern of rhamnolipid congeners synthesised by this strain was highly similar to the rhamnolipid congeners that have been shown to be produced by both *P. aeruginosa* and marine *Pseudomonas sp.* MCTG214(3b1), but contrasts with *B. thailandensis* which produces an abundance of congeners with di-rhamnolipid containing C_{14} [18, 20, 23]. The composition of rhamnolipid congeners greatly affects its properties. Mono-rhamnolipids have been reported to more effectively solubilise PAHs compared to di-rhamnolipids. However, di-rhamnolipids have better rate of biodegradation than mono-rhamnolipids due to the slow release of PAH from the mono-rhamnolipid micelles [53]. This was seen with methyl esters of di-rhamnolipid which were reported to be effective in promoting alkane degradation [54]. Interestingly, non-ionic rhamnolipids or methyl esters of di-rhamnolipid C_{10}-C_{10} and novel methyl ester of di-rhamnolipid C_{10}-C_{12} were also identified in this study. In this study a mono-rhamnolipid with single 3-hydroxy fatty acid chain Rha-$C_{14:2}$ (3.18%) was also detected which was previously reported in rhamnolipid produced by *P. aeruginosa* mutant MIG-N146 [55]. Under our experimental conditions, MCTG107b

was able to produce diverse rhamnolipid congeners with aliphatic chains varying from C_8 to C_{14} and few congeners with unsaturated bonds.

The biosynthesis of rhamnolipids in *P. aeruginosa* occurs in three enzymatic steps. In the first step, rhamnosyltransferase chain A RhlA (encoded by the *rhlA* gene), synthesizes a fatty acid dimer molecule from β-hydroxy fatty acid precursors [56]. The second step, RhlB rhamnosyltransferase chain B (encoded by the *rhlB* gene), produces mono-rhamnolipids by covalently bonding the previously synthesised precursor molecule and dTDP-L-rhamnose [57]. The final step, RhlC rhamnosyltransferase II (encoded by *rhlC* gene), utilises mono-rhamnolipids synthesised by RhlA and RhlB as a substrate, adding a second dTDP-L-rhamnose moiety to produce di-rhamnolipids [58]. The rhamnolipid congener profile produced by *Marinobacter* sp. MCTG107b is predominantly skewed toward the synthesis of di-rhamnolipids, whereas mono-rhamnolipids were only found in much smaller concentrations. Although a prevalence toward di-rhamnolipid synthesis has been observed in *P. aeruginosa*, and to an even greater extent in *Burkholderia* species, the abundance of di-rhamnolipid versus mono-rhamnolipid in *Marinobacter* sp. MCTG107b was significantly higher than that previously observed with other rhamnolipid-producing organisms. In *P. aeruginosa* a single copy of *rhlA* and *rhlB* are located in an operon alongside genes encoding an AHL-mediate quorum sensing system. The *rhlC* gene is located approx. 1 Mbp down stream of this operon [51]. A contrasting arrangement was observed in *B. thailandensis* which has two identical and functional operons containing orthologues of each rhamnolipid synthesis gene (*rhlA*, *rhlB* and *rhlC*), possessing only 40% sequence similarity to those of *P. aeruginosa* [23, 59]. Therefore, *Burkholderia* species can simultaneously express

rhlB and *rhlC* favouring the immediate addition of the second rhamnosyl group to the produced mono-rhamnolipid [59].

We therefore postulate that a biosynthetic pathway similar to that observed in *Burkholderia* might be present here and accounting for the higher ratio of di-rhamnolipid to mono-rhamnolipid congeners. The biosynthetic pathway of rhamnolipid synthesis by *Marinobacter* sp. has however presented a paradox. Although not shown here, we have carried out additional PCR screening for *rhlA*, *rhlB* and *rhlC* using degenerate primers designed from multiple sequence alignments of both *P. aeruginosa* and *B. thailandensis* sequences. To date, ~~This~~ this approach has failed to amplify any DNA sequence which could be involved in ~~identify any~~ rhamnolipid synthesis gene orthologues. Our finding that other similar *Marinobacter* strains fail to synthesise rhamnolipids ~~The absence of orthologues to the *rhl* genes among non-rhamnolipid producing species~~ suggests that the acquisition of rhamnolipid biosynthesis genes by *Marinobacter* sp. MCTG107b may have occurred ~~*rhl* genes occurs~~ through lateral gene transfer from an unrelated ~~a~~ rhamnolipid producing species, as has been observed previously in other rhamnolipid synthesising bacteria [60]. However, based on the un-relatedness of *Marinobacter* sp. MCTG107b to previously reported rhamnolipid producers and the significant sequence differences between the *P. aeruginosa* and *B. thailandensis* synthases, we conclude that rhamnolipid synthesis in this strain is being catalysed by enzymes with significantly different peptide sequences to either of these other species. This sequence difference is also present at the genetic level, accounting for the reasons why our screening protocol failed [23, 59]. Additionally, the observation of mono-rhamnolipid congeners only possessing a single fatty acid side chain being synthesised by this strain corroborates this conclusion since RhlA in both *P.*

aeruginosa and *Burkholderia* species utilises fatty acid dimers as a substrate for rhamnolipid synthesis [56, 59]. To further investigate ~~this~~ the mechanisms of rhamnolipid biosynthesis, we recently obtained the complete genome sequence of *Marinobacter sp*. MCTG107b. We are therefore, currently in the process of carrying out comparative genomic analysis with various other ~~, we are currently combining comparative sequence analysis with whole genome sequencing of various~~ *Marinobacter* strains, with the aim of identifying ~~to identify~~ putative genetic~~gene~~ candidates for rhamnolipid biosynthesis.

Conclusions

Marinobacter sp. MCTG107b, isolated from the marine environment, has the ability to synthesise a wide variety of rhamnolipid congeners. To the best of our knowledge, rhamnolipid production has not been previously observed in any member of the genus *Marinobacter*. Therefore, the results presented here expand the list of known rhamnolipid producing bacterial taxa to include *Marinobacter*; a genus of marine bacteria that shows little to no association with human pathogenicity. Although the major hurdle of low production yield remains, synthesis of rhamnolipids from novel, non-pathogenic marine species, such as *Marinobacter* sp. MCTG107b, is promising for the scale-up in bioprocessing industry or to provide genetic resources for metabolic engineering for the production of specific rhamnolipid congeners.

Additional file 1. Fig. S1. Effect of various sea salt concentrations, pH, temperature and nitrogen source on the growth of *Marinobacter* sp. MCTG107b over a period of 96h. Growth curves show the OD_{600} of strain MCTG107b during growth at different (A)

salinities (5.0 to 40 g/L^{-1}), (B) pH values (4.0 to 8.5), or (C) temperatures (25 °C~~e C~~, 28 °C~~e C~~, 30 °C~~e C~~, 37 °C~~e C~~).

Fig. S2. HPLC-MS chromatogram for rhamnolipids produced by *Marinobacter* sp. MCTG107b. The MS was operated in the negative mode. Main intensities in the chromatogram were Rha-Rha-C_{10}-C_{10} and Rha-Rha-C_{10}-C_{10}-CH_3.

Table S1. Full 2^2 factorial design with pH and salt as independent variables using surface tension and biosurfactant yield as response variables. Surface tension and biosurfactant yield according to full factorial design after 96 h of shake-flask study of *Marinobacter* sp. MCTG107b.

Table S2. Analysis of variance (ANOVA) for response variables surface tension and biosurfactant yield by *Marinobacter* sp. MCTG107b

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Author's contributions

LT designed the experiments, executed experimental work, analyzed data and drafted the manuscript. MST performed phylogenetic identification, ~~and~~ virulence assessment of the strain and assisted with the drafting of the manuscript. AZ performed the HPLC-MS analysis. KS assisted in phenotypic screening of strains. TG, VUI, and GAS

assisted in reviewing the manuscript. RM and IMB designed and supervised the studies. All authors read and approved the final manuscript.

Availability of data and materials

All data generated or analysed during this study are included in this published article and its additional files.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Abbreviations

PAHs, polycyclic aromatic hydrocarbons; MEOR, microbial enhanced oil recovery; *β*-OH-FA, *β*-hydroxy fatty acids; DoE, design of experiments; FFD, full factorial design; gDNA, genomic DNA; EI, emulsification index; SPE, solid phase extraction; HPLC-MS, high performance liquid chromatography-mass spectrometry; NMR, Nuclear Magnetic Resonance

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Tables

TABLE 1. Phylogenetic identification and biosurfactant phenotypic screening results for each bacterial strain. Bacteria were identified by 16S rDNA gene sequencing. Surface tension values (mNm^{-1}) and EI_{24} (%) were obtained from cell-free supernatant samples of cultures incubated for 96 h. ***** ST and **†** EI 24 h of sterile ZM/1 medium was 58 mNm^{-1} and 0%, respectively.

Strain	**Origin**	**BLASTn Identification** (Against NCBI database)	**GeneBank Accession Number**	**Sequence Similarity**	**Phenotypic Screening**	
					ST (mNm^{-1}) *****	$EI_{24\,h}$ (%) **†**
MCTG106	Coastal surface water, Oregon, Washington State, USA	*Marinobacter* sp. NP1383C-30R	MK894600	100%	54.63 ±3.5	40 ± 1.5
MCTG4b	Laboratory culture of *Thalassiosira weissflogii* strain CCMP 1052 isolated from Oslo Fjord, Norway	*Marinobacter* sp. Set72	MK894835	99%	38.5 ± 0.6	42 ± 2.0
MCTG167	Phytoplankton net tow, Oban, UK	*Marinobacter* sp. T23	MK894854	100%	61.55 ± 0.1	N/A
MCTG161(2c3)	Phytoplankton net tow, Oban, UK	*Marinobacter adhaerens* HP15	MK894872	99%	60.0 ± 0.5	45 ± 2.0
MCTG107b	Coastal surface water, Oregon, Washington State, USA	*Marinobacter* sp. R-28768	MK578516	100%	31.0 ± 0.5	40 ± 1.8

TABLE 2. Composition of rhamnolipid congeners synthesised by *Marinobacter* sp. MCTG107b. Rhamnolipid congeners were identified via HPLC-MS in SPE purified extracts from cell-free culture supernatant samples obtained after 96 h growth in a bioreactor.

RT min	*m/z* value	Compound	Mw (Da)	Molecular Form	Relative %
Mono-rhamnolipid congeners					
14.8	387.22	Rha-$C_{14:2}$	386.48	$C_{20}H_{34}O_7$	3.18
21.5	533.46	Rha-C_{10}-C_{12} / Rha-C_{12}-C_{10}	532.71	$C_{28}H_{52}O_9$	0.22
24.2	503.47	Rha-C_{10}-$C_{10:1}$	502.64	$C_{26}H_{46}O_9$	0.27
26.9	561.52	Rha-C_{12}-C_{12}/ Rha-C_{10}-C_{14}	560.76	$C_{30}H_{56}O_9$	0.94
Subtotal					4.61
Di-rhamnolipid congeners					
4.6	453.27	Rha-Rha-C_8	452.49	$C_{20}H_{36}O_{11}$	1.95
12.7	480.39	Rha-Rha-C_{10}	480.55	$C_{22}H_{40}O_{11}$	5.13
22.1	537.45	Rha-Rha-C_{14}	536.65	$C_{26}H_{48}O_{11}$	0.21
31.0	649.71	Rha-Rha-C_{10}-$C_{10:1}$ / Rha-Rha-$C_{10:1}$-C_{10}	648.74	$C_{32}H_{56}O_{13}$	2.85
32.1	651.73	Rha-Rha-C_{10}-C_{10}	650.79	$C_{34}H_{58}O_{13}$	52.45
32.8	677.77	Rha-Rha-C_{10}-$C_{12:1}$	676.83	$C_{33}H_{60}O_{13}$	1.06
33.0	665.77	Rha-Rha-C_{10}-C_{10}-CH_3	664.82	$C_{42}H_{60}O_{13}$	23.07
34.5	803.54	Decenoyl-Rha-Rha-C_{10}-$C_{10:1}$	801.01	$C_{35}H_{72}O_{11}$	0.40
35.1	679.78	Rha-Rha-C_{10}-C_{12} / Rha-Rha-C_{12}-C_{10}	678.84	$C_{35}H_{64}O_{13}$	5.01
37.2	693.90	Rha-Rha-C_{10}-C_{12}-CH_3 / Rha-Rha-C_{12}-C_{10}-CH_3	692.80	$C_{35}H_{64}O_{13}$	3.26
Subtotal					95.39

Figure Legends

Fig 1. Three-dimensional response surface plot modelling the effect of varying media pH and salt concentration on (**A**) biosurfactant yield and (**B**) cell-free supernatant surface tension. The different coloured areas of these plots represent various bands for either predicated yield or predicted surface tension. The values of each band are provided in the key next to each panel.

Fig 2. Biomass and surface tension reduction kinetics of *Marinobacter* sp. MCTG107b during growth under optimised conditions using 1% (v/v) rapeseed oil as a carbon source in a 5L bioreactor. Surface tension (□) was seen to reduce to a stable value within the first 24 h of growth and corresponded with the strain reaching the stationary growth phase, as measured by viable cell counts (○).

Fig 3. HPLC-MS-MS profile of daughter products resulting from the fragmentation of a molecular ion with an *m/z* of 651.73, observed in a previous HPLC-MS analysis to be the predominant compound in supernatant extracts from *Marinobacter* sp. MCTG107b. The observed products corresponded to the predicted molecular weights of the fragmentation of di-rhamnolipid Rha-Rha-C_{10}-C_{10}. Fragments below *m/z* 205 were not detected due to sensitivity of the instrument.

Fig 4. Kaplan-Meier plot showing percentage survival of *Galleria mellonella* larvae after inoculation with either *Marinobacter* sp. MCTG107b or *P. aeruginosa* PAO1. Within a 48 h incubation there was no significant mortality observed after infection with cells of strain MCTG107b as opposed to infection with strain PAO1 where 100% mortality was observed following 24 h incubation. Additionally, no significant mortality was observed in larvae inoculated with the carrier control buffer (PBS). n = 30 (pooled from 3 X duplicate experiments).

Figures

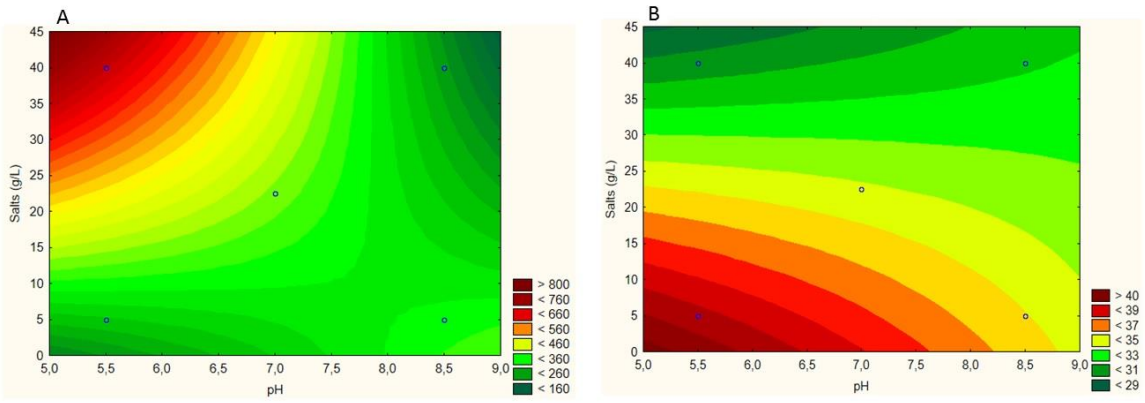

Fig. 1

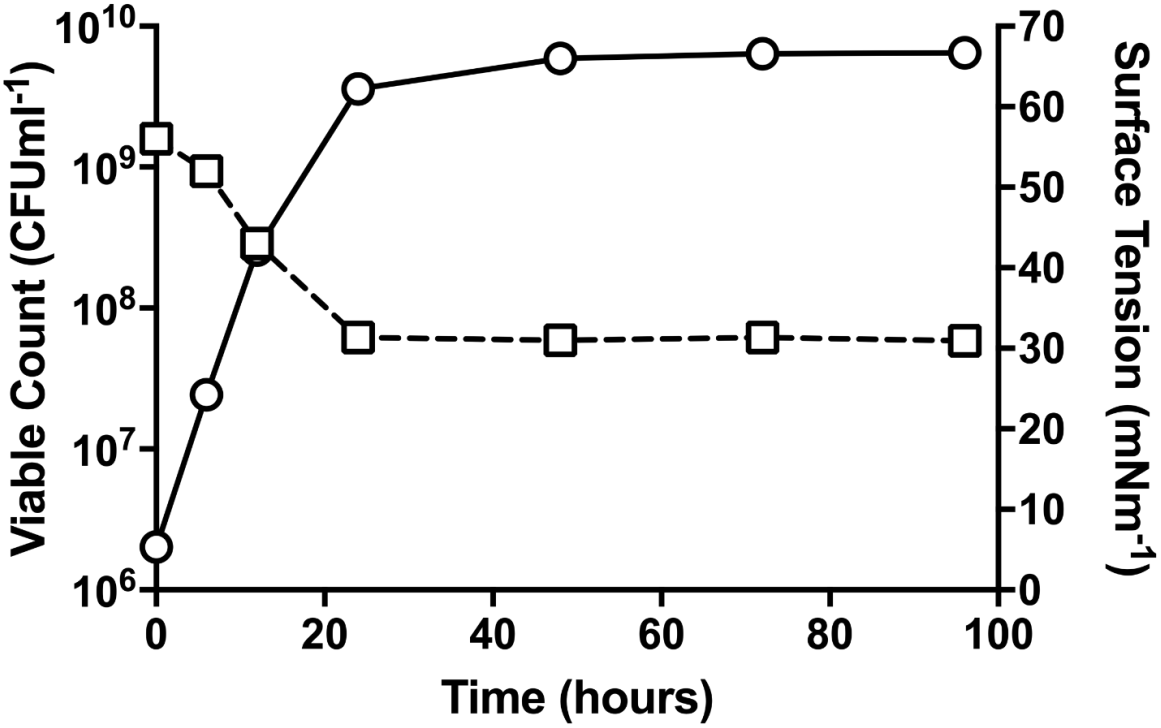
Viable Count (CFUml-1)
10^10
10^9
10^8
10^7
10^6
0
20
40
60
80
100
Time (hours)
70
60
50
40
30
20
10
0
Surface Tension (mNm-1)

Fig. 2

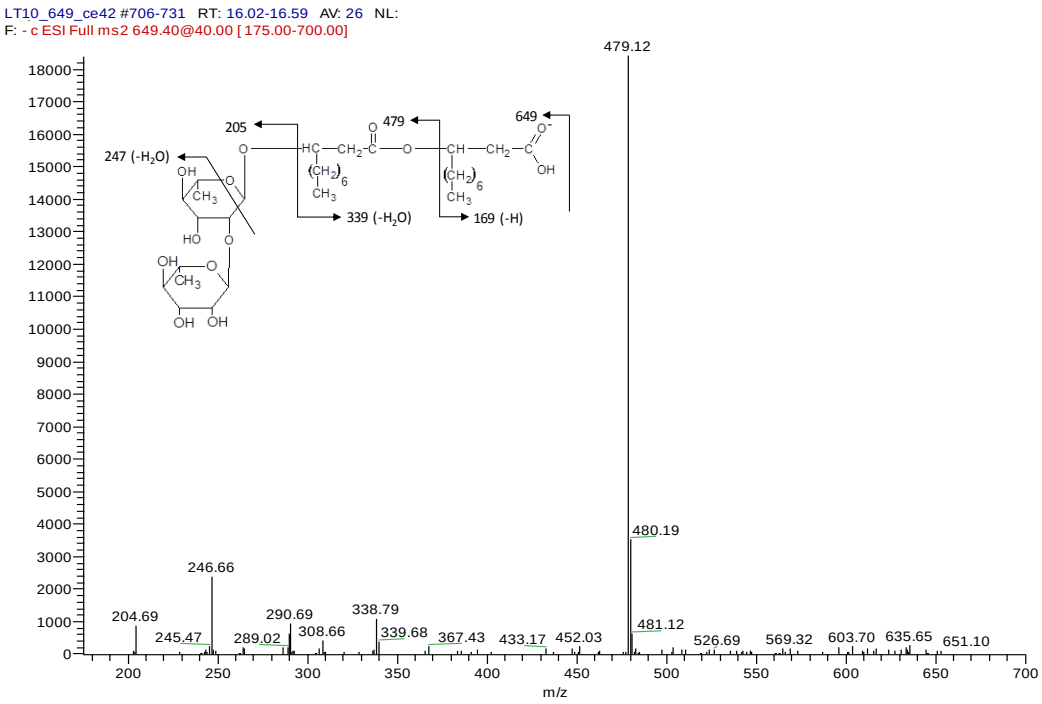
LT10_649_ce42 #706-731 RT: 16.02-16.59 AV: 26 NL:
F: - c ESI Full ms2 649.40@40.00 [175.00-700.00]
479.12
480.19
246.66
204.69
290.69
338.79
481.12
245.47
289.02
308.66
339.68
367.43
433.17
452.03
526.69
569.32
603.70
635.65
651.10
205
479
649
247 (-H2O)
339 (-H2O)
169 (-H)
m/z

Fig. 3

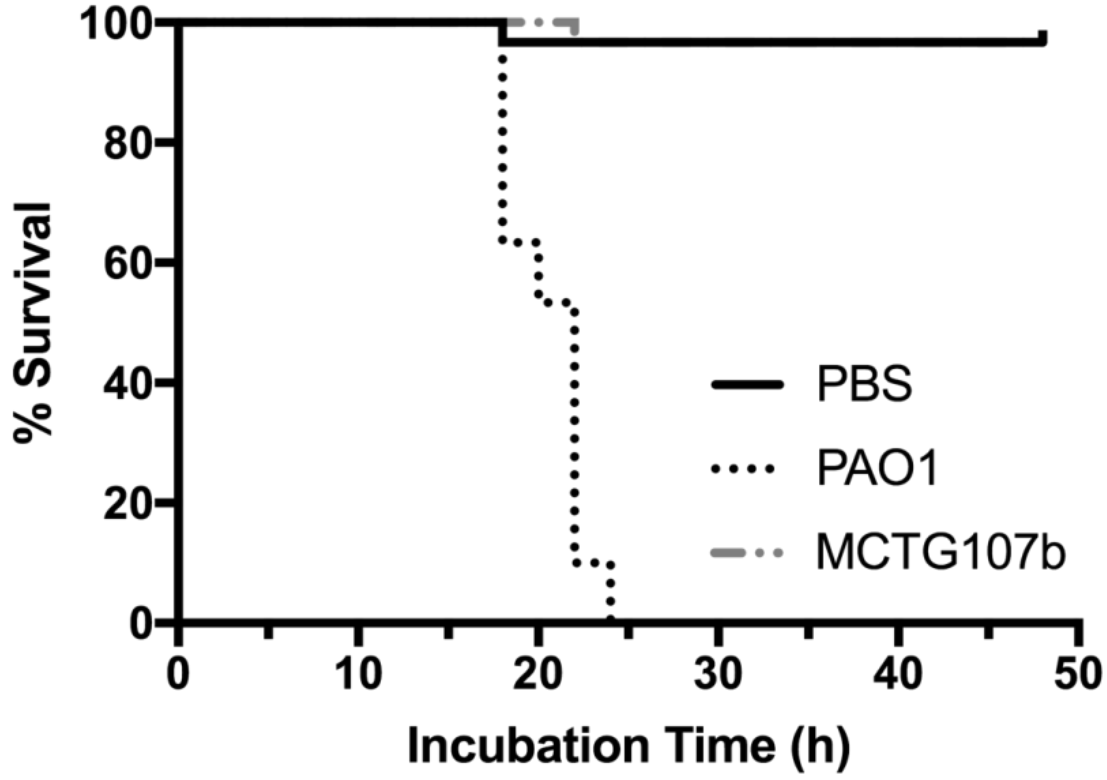
100
80
60
40
20
0
% Survival
0
10
20
30
40
50
Incubation Time (h)
PBS
PAO1
MCTG107b

Fig. 4

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Friday, 6 September 2019

To: Norma A. Valdez-Cruz
Associate Editor
Microbial Cell Factories

Dear Dr. Norma,

Thank you for your efforts to handle our MS (MICF-D-19-00270) entitled “Biosynthesis of rhamnolipid by a *Marinobacter* species expands the paradigm of biosurfactant synthesis to a new genus of the marine microflora.”

We are grateful to you and the reviewers who spent their precious time to raise comments and to help us improve the quality of our MS. We have made point-by-point response and revised/added the additional information as suggested. All changes made in the manuscript is marked by track changes.

Again, thank you for your efforts and I look forward to hearing from you very soon.

Sincerely yours,

Dr. Lakshmi Tripathi
University of Ulster